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Immunocytochemical labelling of aerobic and hypoxic mammalian cells using a platinated derivative of EF5

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Summary The monoclonal antibody ELK3-51 was previously developed to detect adducts of the 2-nitroimidazole EF5. Direct immunofluorescence was used to detect adducts of EF5 or of a platinated derivative *cis*-[$PtCl_2(NH_3)$EF5] in SCCVII cells treated under aerobic or hypoxic conditions. Fluorescence measurements of these cells using both image and flow cytometric methods were compared, giving similar profiles. Platination significantly decreased immunofluorescence levels (~4-fold less than EF5) after 3 h in hypoxia, but also increased levels after exposure in air (~1.5 ×) such that the hypoxic ratio decreased from ~50 to ~13. Platinated EF5 also showed significantly greater cytotoxicity than its parent in both aerobic and hypoxic cells. These results are consistent with targeting of EF5 to DNA, which was confirmed qualitatively by confocal microscopy.

Keywords: DNA targeting; nitroimidazole; hypoxia; image cytometry

It is a widely accepted fact that regions of low oxygen tension exist in many solid tumours (e.g. Cater and Silver, 1960; Vaupel *et al.*, 1991) and there is some evidence for the importance of hypoxia with respect to clinical outcome (Overgaard, 1992). However, the lack of a routine method for assessment of radiobiological hypoxia in human tumours has been a significant problem. Various approaches have been reviewed (Chapman 1991; Stone *et al.*, 1993) including measurement using agents which are reduced and then bind to cellular macromolecules. These adducts may be detected by a variety of methods (i.e. positron emission tomography, ^{125}I SPECT and ^{19}F magnetic resonance imaging, immunohistochemical staining, ELISA, FACS).

Many 2-nitroimidazoles have oxygen-dependent binding properties appropriate for their use as diagnostic indicators of hypoxia, one of which has recently been coupled with a monoclonal antibody specific for their adducts. In particular, ELK3-51, raised against adducts of EF5 (2-[2-nitro-1H-imidozyl-1-yl]-*N*-(2,2,3,3,3,-pentafluoropropyl)acetamide), a pentafluorinated analogue of etanidazole has been used to demonstrate hypoxic cells in culture (Lord *et al.*, 1993; Koch *et al.*, 1995; Woods *et al.*, 1996) and animal tumour models (Lord *et al.*, 1993; Evans *et al.*, 1995; Laughlin *et al.*, 1996). This antibody-drug pair has several properties which make it highly suitable as an hypoxia measurement tool allowing highly sensitive detection of oxygen concentration in individual cells.

Nitroimidazoles have been widely studied for their suitability as hypoxic cytotoxins and radiosensitisers. In general, nitroimidazoles do not selectively bind to DNA, but it is possible to target them to DNA using platinum (Pt) (Skov *et al.*, 1987). DNA binding and hence cytotoxicity and radiosensitising properties of the series [$PtCl_2(NH_3)$(L)] (L = nitroaromatic) is dependent upon the nature of the L ligand (Skov *et al.*, 1987, 1994). These compounds are typically more toxic than the parent nitroimidazoles. Surprisingly, cisplatin itself exhibits some hypoxic selectivity *in vitro* as a cytotoxin (Matthews *et al.*, 1993). *Cis*-[$PtCl_2(NH_3)$EF5] (PtEF5), a platinated complex of EF5, has been synthesised; its cytotoxic effects and immunocytochemical detection using the ELK3-51 antibody were studied. Here we attempt to use this new drug and detection system to characterise further the DNA targeting effects of platinum on nitroimidazole complexes, under both aerobic and hypoxic conditions, and compare quantitatively two methods of immunohistochemical staining measurement, image cytometry and flow cytometry.

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Materials and methods

Drugs

EF5 was synthesised at SRI International, CA, USA (SR4555). The platinum complex, PtEF5 was synthesised according to previously published methods for this series of compounds (Rochon *et al.*, 1991). EF5 and PtEF5 stock solutions were prepared just before each experiment by dissolution in phosphate-buffered saline (PBS) to concentrations of 10 and 1 mM respectively.

Drug treatments

Mouse squamous cell carcinoma SCCVII cells were grown in monolayer cultures in α-MEM supplemented with 10% fetal bovine serum, at 37°C in a 95% air, 5% carbon dioxide atmosphere and passaged twice weekly before confluence was reached. Immediately before each experiment cells were trypsinised and placed in drug-containing medium pregassed for 1 h with either oxygen-free nitrogen or air. Treatments continued for 3 h at 37°C in a shaker bath. Following treatment, cytotoxicity was assessed as described previously, as were Pt accumulation and DNA binding measured by atomic absorption (Matthews *et al.*, 1993).

Immunocytochemical labelling

Control and treated cells were washed twice in ice-cold PBS supplemented with 2.5 mM sodium azide and 0.25 mM thimerosal ('PBS') and stained in a similar fashion to previously published methods (Koch *et al.*, 1995). Briefly, cells were fixed in 4% paraformaldehyde in PBS for 60 min at 4°C and rinsed three times in PBS. Cell pellets were resuspended in a PBS-based blocking solution with 1.5% bovine serum albumin, 20% fresh skim milk and 5% normal mouse serum such that the cell concentration was

2×10^6 ml^{-1}. After 5 h blocking, cells were washed with PBS containing 0.3% Tween 20 (PBS-tt; Sigma) and resuspended in a 1:10 or 1:20 dilution of the primary antibody,which had been directly conjugated with a fluorochrome (Cy3-conjugated ELK3-51), in PBS-tt with 1.5% albumin. After overnight staining at 4°C, cells were washed three times (40 min each) with PBS-tt. Immunostained cells were stored in PBS with 1% paraformaldehyde until analysis by FACS or image cytometry.

Image cytometry (ICM)

A droplet of cell suspension was placed on each microscope slide and coverslips were mounted using 90% glycerol–10% PBS supplemented with *n*-propyl gallate as an anti-fading agent. The image cytometry device used for quantifying immunofluorescence signals was a fluorescence-adapted CytoSavant system (Xillix Technologies Corp., Richmond, BC, Canada). The microscope is equipped with an epifluorescence illumination attachment for Koehler illumination, and uses a 100 W Hg arc lamp with stabilised power supply. Images are captured by a 12-bit scientific CCD camera (MicroImager 1400, Xillix) mounted in the primary image plane, and are stored digitally on an interfaced computer workstation. All measurements were made with a Nikon plan-apochromat 20× objective (N = 0.75) and an additional 1.25× lens within the light path, giving overall magnification of 25×. Cy3 fluorescence was observed using a 510–560 nm excitation filter, 580 nm dichroic mirror and 590 nm high pass barrier filter. For each slide 100 cells were chosen, focused and thresholded to determine cell boundaries and the integrated optical intensity (IOI) of each (sum of fluorescence intensity of all pixels within the cell) was calculated. All IOI measurements were calibrated against a uniformly fluorescent field to remove illumination non-uniformities. Some samples were also viewed at 60× magnification with a BioRad MRC-600 confocal microscope with a krypton–argon laser and BHS filter block (488 nm excitation; 515 nm barrier) to assess staining distribution patterns qualitatively in 0.1 μM slices through the cells. Images obtained with the microscope were stored digitally on disk immediately after acquisition.

Flow cytometry (FCM)

All measurements were performed on a Coulter Epics Elite fluorescence activated cell sorter equipped with a coherent Inova 90 laser. Signals were excited at 510 nm and collected using a 580–590 bandpass filter. Ten thousand cells were measured per data point, gated on forward-and side-scatter signals.

Results

Characterisation of PtEF5

The NMR, mass spectrometry etc. are consistent with the assignment of structure similar to Rochon *et al.*, 1991; this would leave the antigenic pentafluorinated tail distant from the platinum, and the nitro group relatively accessible. Platination of EF5 changed the λ_{max} from 323 to 309 nm, and decreased the extinction coefficient (by ~50%). The NMR of EF5 alone shows some splitting of the imidazole Hs due to the fluorines, but this is absent in the Pt complex. The complex, readily soluble in biological medium (>1 mM as determined by atomic absorption) is significantly more toxic than EF5 (Table I). The hypoxic selectivity of the complex was assessed in survival experiments, from which cells were also examined for the extent of Pt accumulation in cells, and the DNA binding. Table I summarises these data in comparison with cisplatin.

ELK3-51 immunofluorescence

The ELK3-51 antibody raised against EF5 adducts was found to recognise intracellular binding of PtEF5. The dilution factor of the primary antibody was optimised for both EF5 and PtEF5 (100 μM) giving a plateau at a factor of 20. Table II summarises immunofluorescence measurements performed on PtEF5-and EF5-treated cells, detected by the ELK3-51 technique. Under hypoxic conditions, significantly higher mean immunofluorescence intergrated optical intensity ($\overline{IOI}$) was observed in EF5-treated cells compared with PtEF5-treated at equimolar concentrations. $\overline{IOI}$ in aerobically treated cells was consistently larger for PtEF5-treated cells over all four sets of slides from two separate experiments although this is not reflected statistically in the mean values. EF5 treatment resulted in a significantly larger differential $\overline{IOI}$ between air and hypoxia-treated cells than did PtEF5 treatment. Measurements by flow and by image cytometry exhibit very good agreement. Differences in staining patterns between EF5 and PtEF5 seen with ICM were further characterised using confocal microscopy, confirming that with EF5, ELK3-51 appears to be located primarily near the

Table I Cytotoxicity, accumulation and DNA-binding of PtEF5 compared with EF5 and cisplatin. Cytotoxicity is represented as the concentration × time required to reduce plating efficiency (PE) to 0.1, calculated by interpolation from corresponding survival curves. Accumulation and binding studies (in mol drug per mol DNA) used 100 μM PtEF5 for 3 h, or 20 μM cisplatin for 3 h and Pt was determined using atomic absorption (all in SCCVII cells). Cisplatin data are taken from Matthews *et al.*, 1993; all others are the mean of three experiments

	Concentration × time (μM-h) (for PE=0.1)		*Accumulation (μM per cell)*		*DNA binding (M/M)*	
	Air	N_2	*Air*	N_2	*Air*	N_2
PtEF5	168	143*	4.39×10^{-10}	5.19×10^{-10}	4.56×10^{-5}	5.71×10^{-5}
Cisplatin	12.4	9.1**	1.67×10^{-10}	1.86×10^{-10}	1.07×10^{-4}	1.27×10^{-4}
EF5	2320	1630	–	–	–	

*, ~400 and **, ~20 in CHO cells

Table II ELK3-51 mean integrated optical intensity measurements ($\overline{IOI} \pm$ s.e.) in PtEF5- and EF5-treated SCCVII cells as measured by quantitative image (100 cells per slide) and flow cytometry (10 000 cells per sample) techniques (ICM and FCM)

	$\overline{IOI}$ *(ICM)*			$\overline{IOI}$ *(FCM)*		
	Air	N_2	*Ratio*	*Air*	N_2	*Ratio*
PtEF5 (100 μM)	7.13 ± 0.71	90.0 ± 18.4	12.2 ± 1.4	0.618 ± 0.412	7.4 ± 4.1	13.8 ± 2.3
EF5 (100 μM)	5.44 ± 1.06	325.5 ± 10.9	78.2 ± 14.5	0.402 ± 0.212	34.4 ± 25.8	71.8 ± 26.4
Control	1.92 ± 0.39	2.39 ± 0.75	1.16 ± 0.21	0.256 ± .097	0.327 ± 0.038	1.56 ± 0.73

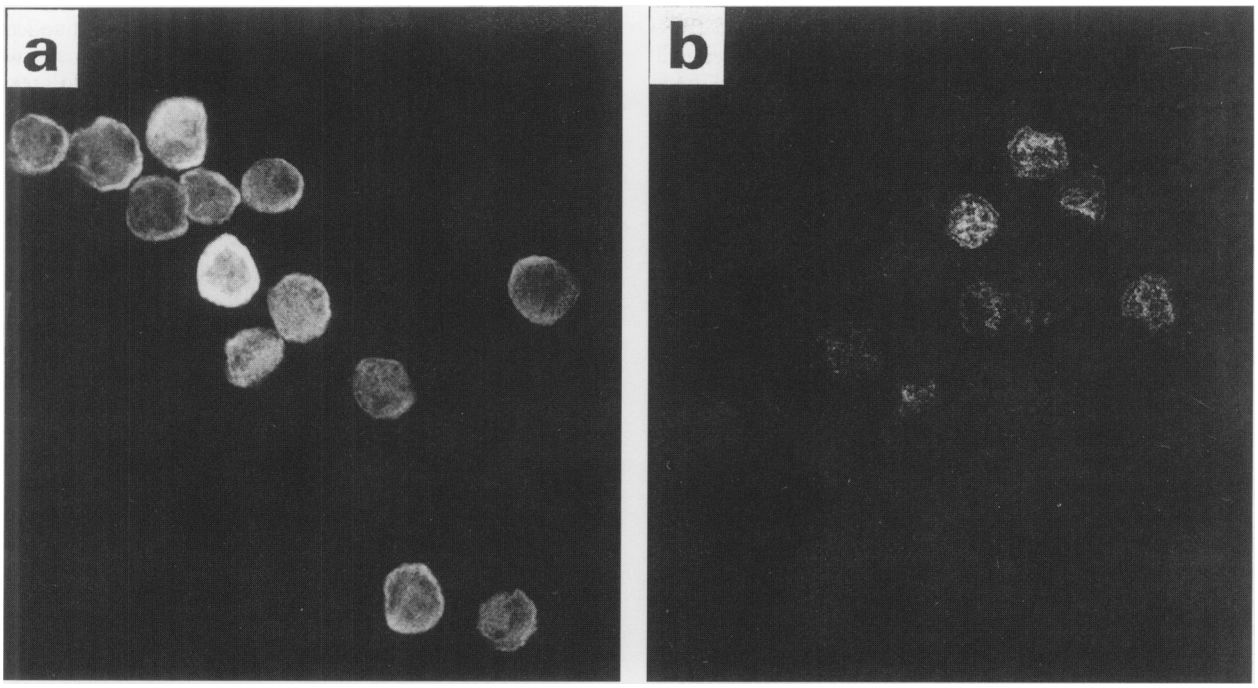

Figure 1 Differences in staining distributions observed by ICM were further studied by confocal microscopy. (**a**) EF5- and (**b**) PtEF5-treated hypoxic cells revealed the DNA-targeting effects of platination.

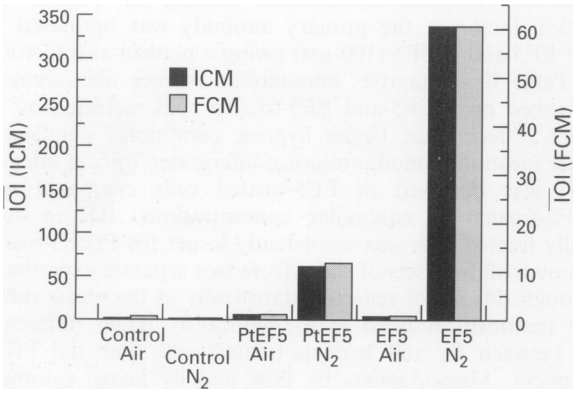

Figure 2 Typical mean ELK3-51 immunofluorescence results due to EF5 and PtEF5 treatment (100 μM, 3 hrs) in SCCVII cells in air or hypoxia, as measured by image cytometry (black, left axis) and flow cytometry (grey, right axis).

periphery of cells treated with EF5 whereas in PtEF5-treated cells, a more granular pattern of staining is observed and greater fluorescence is concentrated within the cell nucleus (Figure 1).

Discussion

In an attempt to improve the efficacy of nitroimidazoles as hypoxic radiosensitisers or cytotoxins, many groups have attempted to target these drugs to DNA by various means (Skov, 1989), including platination. The series $[PtCl_2NH_3L]$ gave the most promising Pt approach, yet there has not been direct evidence for the actual presence of the bioreducible portion near the DNA after binding. We postulated that the sensitivity of the EF5/ELK3-51 system could provide a method of investigating the binding nature of this series, which is generally more toxic than the parent nitroimidazole (presumably due to DNA binding) yet less toxic than the other parent, cisplatin. With survival of $\sim$0.1 after a 2 h 100 μM hypoxic treatment, the cytotoxicity of PtEF5 is in the same range as the analogous metronidazole and misonidazole complexes (summarised for CHO cells, Skov *et al.*, 1994) and an experimental control with the etanidazole analogue in the more sensitive SCCVII cells likewise produced similar hypoxic cytotoxicity (H Adomat, unpublished data). Platination shifts the reduction potentials of the nitroimidazoles, upon which their cytotoxicity is dependent (Adams *et al.*, 1979). For example, the $E_{1/2}$ for etanidazole is -384 mV, while a Pt–etanidazole derivative has an $E_{1/2}$ of -204 mV (Rochon *et al.*, 1991). By analogy then, platination of EF5 could result in lower redox potential and therefore lower sensitivity to oxygen concentration.

The ELK3-51 antibody raised against EF5 adducts also appears to recognise adducts of PtEF5. Although Cy3 fluorescence in hypoxic cells is 4- to 5-fold less in PtEF5- compared with EF5-treated cells, some hypoxic selectivity remained. Our results agree well with reported hypoxia–air ratios in the 50–100-fold range for EF5 (Lord *et al.*, 1993; Koch *et al.*, 1995). For PtEF5, this ratio is reduced to approximately 13. There are several possible explanations for this reduced ratio: different redox potentials of PtEF5 and EF5; lower affinity of the antibody for the Pt derivative; DNA-bound EF5 (via Pt) less accessible to nitroreductases or to the ELK3-51 detection. Although the total fluorescence intensity was less for PtEF5, confocal microscopy confirmed a greater ELK3-51 binding in the nucleus of PtEF5-treated cells (Figure 1). It is therefore possible that less enzymatic reduction takes place within the nucleus; alternatively, the platinum itself, due to cytotoxicity or cross-linking behaviour, might prevent detection of a large portion of the adducts. To test this, cells were treated with cisplatin before or concurrent with EF5 exposure; decreased $\overline{IOI}$($\sim$30%) occurred only at very high concentrations of cisplatin (30 μM, 3 h) which cause extreme cytotoxicity and considerably higher Pt–DNA binding than the PtEF5 concentrations studied here (data not shown). In addition, confocal microscopy revealed that cisplatin treatment had no effect on EF5 intracellular distribution (data not shown). Pretreatment with 20 Gy X-rays ($\sim$equitoxic with 30 μM cisplatin) had no effect on EF5 immunofluorescence indicating that PtEF5 cytotoxicity was probably of no consequence.

Interestingly, while EF5 binding measured by ^{14}C labelling shows an approximate 50 times greater accumulation in hypoxic cells than in oxygenated cells (Koch *et al.*, 1995), measurement of the PtEF5 accumulation by atomic absorption for Pt showed much lower hypoxic selectivity (Table I) as did DNA binding of PtEF5 measured by the same method. This might be expected as platinum binds

DNA regardless of oxygenation status. The fact that PtEF5 still exhibits greater immunofluorescence in hypoxia (13-fold) may reflect the need for reduction of the nitroimidazole to maximise affinity for ELK3-51 (C Koch, unpublished results). Although discrepancies between atomic absorption measurements and immunofluorescence measurements might be explained on the basis of possible lability of the PtEF5 complex (upon binding to DNA or upon reduction), it would be difficult to reconcile this with the apparent localisation of the immunofluorescence within the nucleus. Thus, there is significant evidence to suggest that Pt acts as intended and targets the nitroimidazole to DNA.

Immunofluorescence signals were measured using two different techniques, ICM and FCM, which gave similar results over all conditions, (Figure 2) and showed similar interexperiment fluctuations in signal intensity. The coefficients of variation of the optical intensity distributions of measured cells ranged between 40 and 50% for FCM and 40% and 60% for the ICM. While most of the technical problems inherent in the latter have been corrected or controlled for in its design and operation (Poulin *et al.*, 1994), the largest factor contributing to the increased coefficient of variation values is the relatively small sample population studied compared with FCM. Other factors such as mercury lamp instability and glare would have little noticeable impact. Finally, image cytometric methods have several advantages, including the ability to assess intracellular staining distributions, as demonstrated here by differences observed between EF5 and PtEF5 staining patterns.

In summary, antibodies raised against the 2-nitroimidazole EF5 may be used to detect the platinated analogue, PtEF5. Platination of EF5 significantly increased the cytotoxicity of the compound and resulted in less differential cytotoxicity between aerobic and hypoxic cells. There was also less differential adduct formation as recognised by the ELK3-51 antibody and detected by either flow or image cytometry. However, in the light of previous efforts to target nitroimidazole complexes to DNA using platinum, it is encouraging that platination had the desired effect of increasing nuclear localisation of the drug, and justifies further exploration of this approach.

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